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(54) Title: NOVEL CYTOKINE THAT BINDS CD30

(57) Abstract

There is disclosed a polypeptide (CD30-L) and DNA sequences, vectors and transformed host cells useful in providing CD30-L polypeptides. The CD30L-polypeptide binds to the receptor known as CD30, which is found on Hodgkin's Disease tumor cells.

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TITLE

NOVEL CYTOKINE THAT BINDS CD30

BACKGROUND OF THE INVENTION

Hodgkin's Disease is a human lymphoma, the etiology of which is still not well understood. The neoplastic cells of Hodgkin's Disease are known as Hodgkin and Reed-Sternberg (H-RS) cells. CD30 is a 120 kd surface antigen widely used as a clinical marker for Hodgkin's lymphoma and related hematologic malignancies (Froese et al., *J. Immunol.* 139:2081 (1987); Pfreundschuh et al., *Onkologie* 12:30 (1989); Carde et al., *Eur. J. Cancer* 26:474 (1990)). Originally identified by the monoclonal antibody Ki-1, which is reactive with H-RS cells (Schwab et al., *Nature* (London) 299:65 (1982)), CD30 was subsequently shown to be expressed on a subset of non-Hodgkin's lymphomas (NHL), including Burkitt's lymphoma, as well as several virally-transformed lines (human T Cell Lymphotrophic Virus I or II transformed T cells, and Epstein-Barr Virus transformed B cells (Stein et al., *Blood* 66:848 (1985); Andreeson et al., *Blood* 63:1299 (1984)). Indeed, overall, 50% of Hodgkin's lymphomas are EBV^+ (Klein, *Blood* 80:299 (1992)). That CD30 plays a role in normal lymphoid interactions is suggested by its histological detection on a small population of lymphoid cells in reactive lymph nodes, and by induced expression on purified T and B cells following lectin activation (Stein et al., *Int. J. Cancer* 30:445 (1982) and Stein et al., 1985, *supra*).

Cloning and expression of a gene encoding CD30 has been reported and CD30 has been characterized as a transmembrane protein that possesses substantial homology to the nerve growth factor receptor superfamily (Durkop et al., *Cell* 68:421, 1992). Durkop et al. suggest that CD30 is the receptor for one or more as yet unidentified growth factors, and recognize the importance of investigating the existence and nature of such growth factors in order to achieve insight into the etiology of Hodgkin's Disease.

Prior to the present invention, however, no such growth factors or other molecules that bind to the CD30 receptor were known. A need thus remained for identification and characterization of a ligand for CD30.

SUMMARY OF THE INVENTION

The present invention provides a novel cytokine designated CD30-L, as well as isolated DNA encoding CD30-L protein, expression vectors comprising the isolated DNA, and a method for producing CD30-L by cultivating host cells containing the expression vectors under conditions appropriate for expression of the CD30-L protein. CD30-L is a ligand that binds to the Hodgkin's disease-associated antigen CD30 (a cell surface receptor). Antibodies directed against the CD30-L protein or an immunogenic fragment thereof are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b present a cDNA and encoded amino acid sequence for the receptor known as CD30. This sequence was reported by Durkop et al. (*Cell* 68:421, 1992). The signal peptide is underlined and the transmembrane region is designated by a double underline.

Figure 2 presents cDNA and encoded amino acid sequences for a human IgG1 Fc fragment. The Fc fragment was used to prepare a CD30/Fc fusion protein used in screening procedures to isolate CD30-L cDNA.

Figure 3 presents a DNA sequence, and the amino acid sequence encoded thereby, for the coding region of a murine CD30-L cDNA clone, as described in Example 4. The transmembrane region is underlined. Nucleotides are numbered in the left margin; amino acids in the right margin.

Figure 4 presents a partial amino acid sequence for a human CD30-L cDNA clone as described in Example 6. The human (h) sequence is aligned with an N-terminal portion of the murine (m) sequence (amino acids 1-130). The transmembrane region is underlined for the murine sequence and overlined for the human sequence.

Figure 5 presents a DNA sequence, and the amino acid sequence encoded thereby, for the coding region of a human CD30-L cDNA clone, as described in Example 6. The transmembrane region is underlined. Nucleotides are numbered in the left margin; amino acids in the right margin.

Figure 6 presents a DNA sequence, and the amino acid sequence encoded thereby, for the coding region of a murine CD30-L cDNA clone, as described in Example 7. The transmembrane region is underlined. The encoded protein comprises 19 additional amino acids at the N-terminus when compared with the sequence of figure 3.

Figure 7 presents a DNA sequence, and the amino acid sequence encoded thereby, for the coding region of a human CD30-L cDNA clone, as described in Example 7. The transmembrane region is underlined. The encoded protein comprises

19 additional amino acids at the N-terminus when compared with the sequence of figure 5.

DETAILED DESCRIPTION OF THE INVENTION

cDNA encoding a novel polypeptide that can act as a ligand for the Hodgkin's Disease-associated receptor known as CD30 has been isolated in accordance with the present invention. Also provided are expression vectors comprising the CD30 ligand (CD30-L) cDNA and methods for producing recombinant CD30-L polypeptides by cultivating host cells containing the expression vectors under conditions appropriate for expression of CD30-L, and recovering the expressed CD30-L. Purified CD30-L protein is also encompassed by the present invention.

The present invention also provides CD30-L or antigenic fragments thereof that can act as immunogens to generate antibodies specific to the CD30-L immunogens. Monoclonal antibodies specific for CD30-L or antigenic fragments thereof thus can be prepared.

The novel cytokine disclosed herein is a ligand for CD30, a receptor that is a member of the TNF/NGF receptor superfamily. Therefore, CD30-L is likely to be responsible for transducing a biological signal via CD30, which is known to be expressed on the surface of Hodgkin's Disease tumor cells.

One use of the CD30 ligand of the present invention is as a research tool for studying the pathogenesis of Hodgkin's Disease. As described in example 8, CD30-L enhances the proliferation of the $CD30^+$ neoplastic Hodgkin's Disease-derived lymphoma cell line HDLM-2. The HDLM-2 cells are phenotypically T-cell-like. CD30-L did not produce a detectable effect on proliferation or viability of the B-cell-like, $CD30^+$, Hodgkin's Disease-derived lymphoma cell lines KM-H2 and L-428. The CD30-L of the present invention provides a means for investigating the roles that CD30-L and the cognate receptor may play in the etiology of Hodgkin's Disease.

CD30-L exhibited a cytotoxic effect on the $CD30^+$ non-Hodgkin's lymphoma cell line Karpas 299 (see example 8). Thus, CD30-L has potential use as a therapeutic agent.

The CD30 ligand also induces proliferation of T cells in the presence of an anti-CD3 co-stimulus. The CD30-L of the present invention thus is also useful as a research tool for elucidating the roles that CD30 and CD30-L may play in the immune system. The inducible expression of CD30-L on normal T cells and macrophages, and the presence of its receptor on activated T and B cells, is consistent with both autocrine and paracrine effects.

Upregulation of CD30 accompanying EBV, HTLVI and HTLVII transformation also warrants further investigation, and the CD30-L provided herein is

useful in such studies. HTLVI is the proximal cause of adult T cell Leukemia/Lymphoma. EBV has long been associated with Burkitt's lymphoma and nasopharyngeal carcinoma, and, overall, 50% of Hodgkin's lymphomas are EBV+ (reviewed in Klein, 1992, *supra*).

The CD30-L polypeptides of the present invention also may be employed in *in vitro* assays for detection of CD30 or CD30-L or the interactions thereof. Additional cell types expressing CD30 may be identified, for example.

The term "CD30-L" as used herein refers to a genus of polypeptides which are capable of binding CD30. Human CD30-L is within the scope of the present invention, as are CD30-L proteins derived from other mammalian species. As used herein, the term "CD30-L" includes membrane-bound proteins (comprising a cytoplasmic domain, a transmembrane region, and an extracellular domain) as well as truncated proteins that retain the CD30-binding property. Such truncated proteins include, for example, soluble CD30-L comprising only the extracellular (receptor binding) domain.

Isolation of a cDNA encoding murine CD30-L is described in examples 1-4 below. A human CD30-Fc fusion protein was prepared as described in example 1 for use in screening clones in a direct expression cloning procedure, to identify those expressing a protein that binds CD30.

Briefly, total RNA was isolated from a virally transformed human T-cell line designated HUT-102, which has been described by Durkop et al., *supra*, and Poiesz et al. (*PNAS USA* 77:7415-19, 1980). First strand cDNA was prepared using the total RNA as template. DNA encoding the extracellular domain of human CD30 was amplified by polymerase chain reaction (PCR) using primers based on the human CD30 sequence published by Durkop et al., *supra*., and the amplified DNA fragment was isolated. An expression vector comprising the CD30 extracellular domain DNA fused in-frame to the N-terminus of a human IgG1 Fc region DNA sequence was constructed and transfected into mammalian cells. The expressed protein was purified by a procedure that involved use of a protein G column (to which the Fc portion of the fusion protein binds).

Three activated murine helper T-cell lines were screened using a fluorescence activated cell sorting technique, and all three were found to bind a fluorescent derivative of the CD30-Fc protein. A cDNA library was prepared from one of the murine helper T-cell lines. cDNA from this library (in a mammalian expression vector that also replicates in *E. coli*) was transfected into COS-7 (mammalian) cells, for isolation of clones expressing a CD30-binding protein by using a direct expression cloning technique. The clones were screened for ability to bind ^{125}I-CD30/Fc, and a positive clone was isolated. The recombinant vector isolated from the positive clone (murine CD30-L cDNA in plasmid pDC202) was transformed into *E. coli* cells, deposited with

the American Type Culture Collection on May 28, 1992, and assigned accession no. ATCC 69004. The deposit was made under the terms of the Budapest Treaty.

The murine CD30-L cDNA was radiolabeled and used as a probe to isolate human CD30-L cDNA by cross-species hybridization. Briefly, a cDNA library prepared from activated human peripheral blood lymphocytes was screened with ^{32}P-labeled murine cDNA and a positive clone was isolated as described in Example 6. Human CD30-L DNA isolated from the positive clone was inserted into plasmid pGEMBL and then transformed into *E. coli* cells as described in Example 6. Samples of *E. coli* cells transformed with the recombinant vector were deposited with the American Type Culture Collection on June 24, 1992, and assigned accession no. ATCC 69020. The deposit was made under the terms of the Budapest Treaty.

Additional murine and human CD30-L DNA sequences were isolated as described in example 7. The proteins encoded by the clones of example 7 comprise additional amino acids at the N-terminus, compared to the clones isolated in examples 4 and 6.

CD30-L proteins of the present invention thus include, but are not limited to, murine CD30-L proteins characterized by the N-terminal amino acid sequence Met-Gln-Val-Gln-Pro-Gly-Ser-Val-Ala-Ser-Pro-Trp (Figure 3) or Met-Glu-Pro-Gly-Leu-Gln-Gln-Ala-Gly-Ser-Cys-Gly (Figure 6). Human CD30-L proteins characterized by the N-terminal amino acid sequence Met-His-Val-Pro-Ala-Gly-Ser-Val-Ala-Ser-His-Leu (Figure 5) or Met-Asp-Pro-Gly-Leu-Gln-Gln-Ala-Leu-Asn-Gly-Met (Figure 7) also are provided.

While a CD30/Fc fusion protein was employed in the screening procedure described in example 4 below, labeled CD30 could be used to screen clones and candidate cell lines for expression of CD30-L proteins. The CD30/Fc fusion protein offers the advantage of being easily purified. In addition, disulfide bonds form between the Fc regions of two separate fusion protein chains, creating dimers. The dimeric CD30/Fc receptor was chosen for the potential advantage of higher affinity binding of the CD30 ligand, in view of the possibility that the ligand being sought would be multimeric.

Further, other suitable fusion proteins comprising CD30 may be substituted for CD30/Fc in the screening procedures. Other fusion proteins can be made by fusing a DNA sequence for the ligand binding domain of CD30 to a DNA sequence encoding another polypeptide that is capable of affinity purification, for example, avidin or streptavidin. The resultant gene construct can be introduced into mammalian cells to express a fusion protein. Receptor/avidin fusion proteins can be purified by biotin affinity chromatography. The fusion protein can later be recovered from the column by eluting with a high salt solution or another appropriate buffer. Other antibody Fc

regions may be substituted for the human IgG1 Fc region described in example 1. Other suitable Fc regions are defined as any region that can bind with high affinity to protein A or protein G, and include the Fc region of murine IgG1 or fragments of the human IgG1 Fc region, e.g., fragments comprising at least the hinge region so that interchain disulfide bonds will form.

cDNA encoding a CD30-L polypeptide may be isolated from other mammalian species by procedures analogous to those employed in isolating the murine CD30-L clone. For example, a cDNA library derived from a different mammalian species may be substituted for the murine cDNA library that was screened for binding of radioiodinated human CD30/Fc fusion protein in the direct expression cloning procedure described in example 4. Cell types from which cDNA libraries may be prepared may be chosen by the FACS selection procedure described in example 2, or any other suitable technique. As one alternative, mRNAs isolated from various cell lines can be screened by Northern hybridization to determine a suitable source of mammalian CD30-L mRNA for use in cloning a CD30-L gene.

Alternatively, one can utilize the murine or human CD30-L cDNAs described herein to screen cDNA derived from other mammalian sources for CD30-L cDNA using cross-species hybridization techniques. Briefly, an oligonucleotide based on the nucleotide sequence of the coding region (preferably the extracellular region) of the murine or human clone, or, preferably, the full length CD30-L cDNA, is prepared by standard techniques for use as a probe. The murine or human probe is used to screen a mammalian cDNA library or genomic library, generally under moderately stringent conditions.

CD30-L proteins of the present invention include, but are not limited to, murine CD30-L comprising amino acids 1-220 of figure 3 or 1-239 of figure 6; human CD30-L comprising amino acids 1-215 of figure 5 or 1-234 of figure 7; and proteins that comprise N-terminal, C-terminal, or internal truncations of the foregoing sequences , but retain the desired biological activity. Examples include murine CD30-L proteins comprising amino acids x to 239 of figure 6, wherein x is 1-19 (i.e., the N-terminal amino acid is selected from amino acids 1-19 of figure 6, and the C-terminal amino acid is amino acid 239 of figure 6.) As described in example 7, amino acids 1-19 of the figure 6 sequence are not essential for binding of murine CD30-L to the CD30 receptor. Also provided by the present invention are human CD30-L proteins comprising amino acids y to 234 of figure 7 wherein y is 1-19 (i.e., the N-terminal amino acid is any one of amino acids 1-19 of figure 7, and amino acid 234 is the C-terminal amino acid. Such proteins, truncated at the N-terminus, are capable of binding CD30, as discussed in example 7.

One embodiment of the present invention provides soluble CD30-L polypeptides. Soluble CD30-L polypeptides comprise all or part of the extracellular domain of a native CD30-L but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. Since the CD30-L protein lacks a signal peptide, a heterologous signal peptide is fused to the N-terminus of a soluble CD30-L protein to promote secretion thereof, as described in more detail below. The signal peptide is cleaved from the CD30-L protein upon secretion from the host cell. The soluble CD30-L polypeptides that may be employed retain the ability to bind the CD30 receptor. Soluble CD30-L may also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble CD30-L protein is capable of being secreted.

Soluble CD30-L may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired protein from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The culture medium may be assayed using procedures which are similar or identical to those described in the examples below. The presence of CD30-L in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

The use of soluble forms of CD30-L is advantageous for certain applications. Purification of the proteins from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells.

Examples of soluble CD30-L polypeptides include those comprising the entire extracellular domain of a native CD30-L protein. One such soluble CD30-L comprises amino acids 49 (Gln) through 220 (Asp) of the murine CD30-L sequence of Figure 3. Other soluble CD30-L polypeptides comprise amino acids z to 215 (Asp) of the human CD30-L sequence of Figure 5, wherein z is 44, 45, 46, or 47. In other words, the N-terminal amino acid of the soluble human CD30-L is selected from the amino acids in positions 44-47 of Figure 5.

Truncated CD30-L, including soluble polypeptides, may be prepared by any of a number of conventional techniques. In the case of recombinant proteins, a DNA fragment encoding a desired fragment may be subcloned into an expression vector. Alternatively, a desired DNA sequence may be chemically synthesized using known techniques. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. Linkers containing restriction endonuclease cleavage site(s) may be employed to insert the desired DNA fragment into an expression vector, or the fragment may be digested at cleavage sites naturally present therein. The well known polymerase

chain reaction procedure also may be employed to isolate a DNA sequence encoding a desired protein fragment.

In another approach, enzymatic treatment (e.g., using Bal 31 exonuclease) may be employed to delete terminal nucleotides from a DNA fragment to obtain a fragment having a particular desired terminus. Among the commercially available linkers are those that can be ligated to the blunt ends produced by Bal 31 digestion, and which contain restriction endonuclease cleavage site(s). Alternatively, oligonucleotides that reconstruct the N- or C-terminus of a DNA fragment to a desired point may be synthesized. The oligonucleotide may contain a restriction endonuclease cleavage site upstream of the desired coding sequence and position an initiation codon (ATG) at the N-terminus of the coding sequence.

The present invention provides purified CD30-L polypeptides, both recombinant and non-recombinant. Variants and derivatives of native CD30-L proteins that retain the desired biological activity are also within the scope of the present invention. CD30-L variants may be obtained by mutations of nucleotide sequences coding for native CD30-L polypeptides. A CD30-L variant, as referred to herein, is a polypeptide substantially homologous to a native CD30-L, but which has an amino acid sequence different from that of native CD30-L (human, murine or other mammalian species) because of one or a plurality of deletions, insertions or substitutions.

The variant amino acid sequence preferably is at least 80% identical to a native CD30-L amino acid sequence, most preferably at least 90% identical. The degree of homology (percent identity) may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Alterations of the native amino acid sequence may be accomplished by any of a number of known techniques. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting

reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making such alterations are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462, which are incorporated by reference herein.

Variants may comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known.

CD30-L also may be modified to create CD30-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of CD30-L may be prepared by linking the chemical moieties to functional groups on CD30-L amino acid side chains or at the N-terminus or C-terminus of a CD30-L polypeptide or the extracellular domain thereof. Other derivatives of CD30-L within the scope of this invention include covalent or aggregative conjugates of CD30-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a soluble CD30-L polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

CD30-L polypeptide fusions can comprise peptides added to facilitate purification and identification of CD30-L. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK), which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide

may also be resistant to intracellular degradation in *E. coli*. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the peptide DYKDDDDK in the presence of certain divalent metal cations (as described in U.S. Patent 5,011,912) and has been deposited with the American Type Culture Collection under accession no HB 9259.

The present invention further includes CD30-L polypeptides with or without associated native-pattern glycosylation. CD30-L expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native CD30-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of CD30-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding can be prepared. For example, N-glycosylation sites in the CD30-L extracellular domain can be modified to preclude glycosylation while allowing expression of a homogeneous, reduced carbohydrate analog using yeast or mammalian expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate modifications to the nucleotide sequence encoding this triplet will result in substitutions, additions or deletions that prevent attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846.

In another example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other variants are prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites. The resulting muteins are less susceptible to cleavage by the

KEX2 protease at locations other than the yeast α-factor leader sequence, where cleavage upon secretion is intended.

Naturally occurring CD30-L variants are also encompassed by the present invention. Examples of such variants are proteins that result from alternative mRNA splicing events (since CD30-L presumably is encoded by a multi-exon gene) or from proteolytic cleavage of the CD30-L protein, wherein the CD30-binding property is retained. Alternative splicing of mRNA may yield a truncated but biologically active CD30-L protein, such as a naturally occurring soluble form of the protein, for example. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the CD30-L protein (generally from 1-5 terminal amino acids).

Nucleic acid sequences within the scope of the present invention include isolated DNA and RNA sequences that hybridize to the CD30-L nucleotide sequences disclosed herein under conditions of moderate or severe stringency, and which encode biologically active CD30-L. Moderate stringency hybridization conditions refer to conditions described in, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989). Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing. The skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as the length of the probe.

The present invention thus provides isolated DNA sequences encoding biologically active CD30-L, selected from: (a) DNA derived from the coding region of a native mammalian CD30-L gene (e.g., DNA comprising the nucleotide sequence presented in figures 3, 5, 6, or 7; (b) DNA capable of hybridization to a DNA of (a) under moderately stringent conditions and which encodes biologically active CD30-L; and (c) DNA which is degenerate as a result of the genetic code to a DNA defined in (a) or (b) and which encodes biologically active CD30-L. CD30-L proteins encoded by the DNA sequences of (a), (b) and (c) are encompassed by the present invention.

Examples of CD30-L proteins encoded by DNA that varies from the native DNA sequences of Figures 3, 5, 6, and 7, wherein the variant DNA will hybridize to a native DNA sequence under moderately stringent conditions, include, but are not limited to, CD30-L fragments (soluble or membrane-bound) and CD30-L proteins comprising inactivated N-glycosylation site(s), inactivated KEX2 protease processing site(s), or conservative amino acid substitution(s), as described above. CD30-L

proteins encoded by DNA derived from other mammalian species, wherein the DNA will hybridize to the human or murine DNA of Figures 3, 5, 6, or 7, are also encompassed.

Variants possessing the requisite ability to bind CD30 may be identified by any suitable assay. Biological activity of CD30-L may be determined, for example, by competition for binding to the ligand binding domain of CD30 (i.e. competitive binding assays).

One type of a competitive binding assay for CD30-L polypeptide uses a radiolabeled, soluble human or murine CD30-L and intact cells expressing cell surface CD30 (e.g., cell lines such as HUT102, described by Durkop et al., *supra*). Instead of intact cells, one could substitute soluble CD30 bound to a solid phase (such as a CD30/Fc fusion protein bound to a Protein A or Protein G column through interaction with the Fc region of the fusion protein). Another type of competitive binding assay utilizes radiolabeled soluble CD30 such as a CD30/Fc fusion protein, and intact cells expressing CD30-L. Alternatively, soluble CD30-L could be bound to a solid phase.

Competitive binding assays can be performed using standard methodology. For example, radiolabeled murine CD30-L can be used to compete with a putative CD30-L homolog to assay for binding activity against surface-bound CD30. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

Competitive binding assays with intact cells expressing CD30 can be performed by two methods. In a first method, cells expressing cell surface CD30 are grown either in suspension or by adherence to tissue culture plates. Adherent cells can be removed by treatment with 5 mM EDTA treatment for ten minutes at 37° C. In a second method, transfected COS cells expressing membrane-bound CD30 can be used. COS cells or another mammalian cell can be transfected with human CD30 cDNA in an appropriate vector to express full length CD30 with an extracellular region.

Alternatively, soluble CD30 can be bound to a solid phase such as a column chromatography matrix or a similar substrate suitable for analysis for the presence of a detectable moiety such as ^{125}I. Binding to a solid phase can be accomplished, for example, by obtaining a CD30/Fc fusion protein and binding it to a protein A or protein G-containing matrix.

Another means to measure the biological activity of CD30-L (including variants) is to utilize conjugated, soluble CD30 (for example, ^{125}I-CD30/Fc) in competition assays similar to those described above. In this case, however, intact cells expressing CD30-L, or soluble CD30-L bound to a solid substrate, are used to measure competition for binding of labeled, soluble CD30 to CD30-L by a sample containing a putative CD30-L variant.

The CD30-L of the present invention can be used in a binding assay to detect cells expressing CD30. For example, CD30-L or an extracellular domain or a fragment thereof can be conjugated to a detectable moiety such as ^{125}I. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-CD30-L molecule labeled to high specific activity. Alternatively, another detectable moiety such as an enzyme that can catalyze a colorometric or fluorometric reaction, biotin or avidin may be used. Cells to be tested for CD30 expression can be contacted with conjugated CD30-L. After incubation, unbound conjugated CD30-L is removed and binding is measured using the detectable moiety.

CD30-L polypeptides may exist as oligomers, such as dimers or trimers. Oligomers may be linked by disulfide bonds formed between cysteine residues on different CD30-L polypeptides. In one embodiment of the invention, a CD30-L dimer is created by fusing CD30-L to the Fc region of an antibody (IgG1) in a manner that does not interfere with binding of CD30-L to the CD30 ligand binding domain. The Fc polypeptide preferably is fused to the N-terminus of a soluble CD30-L (comprising only the extracellular domain). A procedure for isolating DNA encoding an IgG1 Fc region for use in preparing fusion proteins is presented in example 1 below. A gene fusion encoding the CD30-L/Fc fusion protein is inserted into an appropriate expression vector. The CD30-L/Fc fusion proteins are allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding divalent CD30-L. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a CD30-L oligomer with as many as four CD30-L extracellular regions.

Alternatively, one can link multiple copies of CD30-L *via* peptide linkers. A fusion protein comprising two or more copies of CD30-L (preferably soluble CD30-L polypeptides), separated by peptide linkers, may be produced by recombinant DNA technology. Among the peptide linkers that may be employed are amino acid chains that are from 5 to 100 amino acids in length, preferably comprising amino acids selected from the group consisting of glycine, asparagine, serine, threonine, and alanine. In one embodiment of the present invention, a fusion protein comprises two or three soluble CD30-L polypeptides linked *via* a peptide linker selected from $Gly_4SerGly_5Ser$ and $(Gly_4Ser)_n$, wherein n is 4-12. The production of recombinant fusion proteins comprising peptide linkers is illustrated in United States Patent 5,073,627, for example.

The present invention provides oligomers of CD30-L extracellular domains or fragments thereof, linked by disulfide bonds, or expressed as fusion proteins with or without spacer amino acid linking groups. For example, a dimer CD30-L molecule can be linked by an IgG Fc region linking group. Analysis of expressed recombinant

CD30-L of the present invention by SDS-PAGE revealed both monomeric and oligomeric forms of the protein. The CD30-L proteins of the present invention are believed to form oligomers (disulfide-bonded dimers, trimers and higher oligomers) intracellularly. The oligomers then become attached to the cell surface via the transmembrane region of the protein.

The present invention provides recombinant expression vectors for expression of CD30-L, and host cells transformed with the expression vectors. Any suitable expression system may be employed. The vectors include a CD30-L DNA sequence (e.g., a synthetic or cDNA-derived DNA sequence encoding a CD30-L polypeptide) operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the CD30-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a CD30-L DNA sequence if the promoter nucleotide sequence controls the transcription of the CD30-L DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not native to the CD30-L gene can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in frame to the CD30-L sequence so that the CD30-L is initially translated as a fusion protein comprising the signal peptide. A signal peptide fused to the N-terminus of a soluble CD30-L protein promotes extracellular secretion of the CD30-L. The signal peptide is cleaved from the CD30-L polypeptide upon secretion of CD30-L from the cell. Signal peptides are chosen according to the intended host cells, and representative examples are described below.

Suitable host cells for expression of CD30-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce CD30-L polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the

genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a CD30-L polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant CD30-L polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a CD30-L DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec. Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

CD30-L alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase,

phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the CD30-L polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See*, e.g., Kurjan et al., *Cell 30*:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984; U.S. Patent 4,546,082; and EP 324,274. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant CD30-L polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2,

Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Exemplary expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-A-0367566, and in U.S. Patent Application Serial No. 07/701,415, filed May 16, 1991, incorporated by reference herein. The vectors may be derived from retroviruses. To achieve secretion of CD30 (a type II protein lacking a native signal sequence), a heterologous signal sequence may be added. Examples of signal peptides useful in mammalian expression systems are the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature 312*:768 (1984); the interleukin-4 signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846. Each of these references describing signal peptides is hereby incorporated by reference.

The present invention provides substantially homogeneous CD30-L protein, which may be produced by recombinant expression systems as described above or purified from naturally occurring cells. The CD30-L is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

In one embodiment of the present invention, CD30-L is purified from a cellular source using any suitable protein purification technique. The cells may, for example, be activated T-lymphocytes from a mammalian species of interest, such as the murine cell line 7B9 described in examples 2 and 3 or induced human peripheral blood T-cells.

An alternative process for producing the CD30-L protein comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that

encodes CD30-L under conditions such that CD30-L is expressed. The CD30-L protein is then recovered from culture medium or cell extracts, depending upon the expression system employed. As the skilled artisan will recognize, procedures for purifying the recombinant CD30-L will vary according to such factors as the type of host cells employed and whether or not the CD-30-L is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify CD30-L. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a substantially homogeneous recombinant protein.

It is also possible to utilize an affinity column comprising the ligand binding domain of CD30 to affinity-purify expressed CD30-L polypeptides. CD30-L polypeptides can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use. Alternatively, the affinity column may comprise an antibody that binds CD30-L. Example 5 describes a procedure for employing the CD30-L protein of the present invention to generate monoclonal antibodies directed against CD30-L.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express CD30-L as a secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those

disclosed by Urdal et al. (*J. Chromatog. 296*:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

The present invention further provides antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target CD30-L mRNA (sense) or CD30-L DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of CD30-L cDNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of CD30-L proteins.

Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oliginucleotide for the target nucleotide sequence. Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or other gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, those derived from the murine

retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656).

Sense or antisense oligonucleotides may also be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The following examples are provided to illustrate particular embodiments and not to limit the scope of the invention.

EXAMPLE 1: Preparation of Soluble CD30/Fc Fusion Protein

This example describes construction of a CD30/Fc-encoding vector to express a soluble CD30/Fc fusion protein for use in detecting cDNA clones encoding a CD30 ligand. A cDNA fragment encoding the extracellular region (ligand binding domain) of the CD30 human receptor was obtained using polymerase chain reaction (PCR) techniques, and is based upon the sequence published by Durkop et al. (*Cell* 68:421, 1992) and presented herein in Figure 1.

The CD30 cDNA used as a template in the PCR reaction was prepared as follows. Total RNA was isolated from a virally transformed human T-cell line designated HUT 102E. This cell line was derived by transforming T-cells with human T-cell lymphotropic virus 1 (HTLV-1) as described by Poiesz et al. (*PNAS USA* 77:7415-19, 1980). First strand cDNA was prepared using a SuperScript™ cDNA synthesis kit available from GIBCO/BRL (Gaithersburg, Maryland). The resulting single-stranded cDNA was employed as the template in a PCR reaction.

The 5' primer employed in the PCR reaction was a single-stranded oligonucleotide (39-mer) of the sequence:

5' ATAGCGGCCGCCACCATGCGCGTCCTCCTCGCCGCGCTG 3'

This primer comprises a recognition site for the restriction endonuclease *NotI* (underlined) upstream of a sequence (double underline) encoding the first (N-terminal) eight amino acids of the CD30 sequence shown in Figure 1, from methionine (encoded by the translation initiation codon ATG) through leucine at position eight.

The 3' primer employed in the PCR reaction was a single-stranded oligonucleotide (39-mer) of the sequence:

3' CAGCGAGAGAGGAGGTGCCCCTTCCTCGGGTCTAGAACA 5'

This primer comprises a sequence (double underline) that is complementary to the sequence that encodes the last eight amino acids of the CD30 extracellular domain, i.e., amino acids 372 (Val) through 379 (Lys) shown in Figure 1. The sequence CTCGGG that follows the CD30 sequence is complementary to codons for Glu and Pro. Glu and Pro are the first two amino acids of an antibody Fc fragment that is fused to the C-terminus of the CD30 fragment as described below. The primer also positions a recognition site for the restriction endonuclease *Bgl*II (underlined) downstream, for use in attaching a DNA sequence encoding the remainder of the Fc-encoding gene.

The PCR reaction may be conducted using any suitable procedure, such as those described in Sarki et al., *Science* 239:487 (1988); in *Recombinant DNA Methodology*, Wu et al., eds., Academic Press Inc., San Diego (1989), pp. 189-196; and in *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc. (1990). An example of a suitable PCR procedure is as follows. All temperatures are in degrees centigrade. The following PCR reagents are added to a 0.5 ml Eppendorf microfuge tube: 10 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3 at 25°C, 25 mM $MgCl_2$, and 1 mg/ml gelatin) (Perkins-Elmer Cetus, Norwalk, CN), 8 µl of a 2.5 mM solution containing each dNTP (2 mM dATP, 2mM dCTP, 2mM dGTP and 2 mM dTTP), 2.5 units (0.5 µl of standard 5000 units/ml solution) of *Taq* DNA polymerase (Perkins-Elmer Cetus), 1 ng of template DNA, 100 picomoles of each of the oligonucleotide primers, and water to a final volume of 100 µl. The final mixture is then overlaid with 100 µl parafin oil. PCR is carried out using a DNA thermal cycler (Ericomp, San Diego, CA).

In a preferred procedure, the template was denatured at 94° for 5 minutes, followed by 5 cycles of 94° for 1 minute (denaturation), 48° for 1 min. (annealing), and 72° for 1 min. (extension); followed by 30 cycles of 94° for 1 min., 68° for 1 min., and 72° for 1 min., with the last cycle being followed by a final extension at 72° for 5 mins. An aliquot of the products of this PCR reaction was reamplified in a second PCR reaction, using the same conditions.

The desired DNA fragment amplified by this PCR reaction comprised a *Not*I site upstream of a sequence encoding the entire extracellular domain of CD30, followed by a *Bgl*II site. The PCR reaction products were digested with *Not*I and *Bgl*II, and the desired fragment was purified by gel electrophoresis.

A DNA sequence encoding an antibody Fc fragment, to be fused to the CD30-encoding DNA fragment, was prepared as follows. DNA encoding a single chain polypeptide derived from the Fc region of a human IgG1 antibody has been cloned into

the *Spe*I site of the pBLUESCRIPT SK® vector, which is available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. The DNA and encoded amino acid sequences of the cloned Fc cDNA coding region are presented in Figure 2. A unique *Bgl*II site has been introduced near the 5' end of the inserted Fc encoding sequence as shown in Figure 2.

The Fc polypeptide encoded by the DNA extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate CD30/Fc fusion proteins, forming dimers as discussed above.

The recombinant vector containing the Fc sequence is digested with *Bgl*II (which cleaves only at the site shown in Figure 2) and *Not*I (which cleaves the vector in the multiple cloning site downstream of the Fc cDNA insert. The Fc-encoding fragment (about 720 bp in length) was isolated by conventional procedures using LMT agarose gel electrophoresis.

The *Not*I/*Bgl*II CD30-encoding DNA fragment and the *Bgl*II/*Not*I Fc-encoding DNA fragment prepared above were ligated into an expression vector designated pDC406 as follows. Plasmid pDC406, which has been described by McMahan et al. (*EMBO J.* 10:2821, 1991), is an expression vector for use in mammalian cells, but is also replicable in *E. coli* cells.

pDC406 contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J. Immunol.* 142:4314 (1989). pDC406 differs from HAV-EO by the deletion of the intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. pDC406 was digested with *Not*I, which cleaves the plasmid in a multiple cloning site just 3' of the *Sal*I site, then treated with calf intestine alkaline phosphatase (CIAP) to prevent self ligation.

A three-way ligation to join the vector, Fc, and CD30 DNA fragments was conducted under conventional conditions, and *E. coli* cells were transformed with the ligation mixture. A plasmid of the desired size that was recovered from the *E. coli* cells was found to comprise the CD30/Fc gene fusion insert, but in the wrong orientation for expression. The CD30/Fc gene fusion was excised from this recombinant plasmid by *Not*I digestion and ligated to *Not*I-digested and CIAP-treated pDC406. *E. coli* cells were transformed with the ligation mixture. A recombinant plasmid containing the insert in the desired orientation was isolated. The CD30 sequence was fused (in the same reading frame) to the downstream Fc sequence.

CD30/Fc fusion molecules preferably are synthesized in recombinant mammalian cell culture because they are generally too large and complex to be synthesized by prokaryotic expression methods. Examples of suitable mammalian cells for expressing a receptor/Fc fusion protein include CV-1 cells (ATCC CCL 70) and COS-7 cells (ATCC CRL 1651), both derived from monkey kidney.

The DNA construct pDC406/CD30/Fc was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). In mammalian host cells such as CV1/EBNA, the CD30/Fc fusion protein is expressed off the HIV transactivating region (TAR) promoter. The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line (ATCC CCL 70) with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human CMV intermediate-early enhancer/promoter as described by McMahan et al., *supra*. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

CVI-EBNA cells transfected with the pDC406/CD30/Fc vector were cultivated in roller bottles to allow transient expression of the fusion protein, which is secreted into the culture medium *via* the CD30 signal peptide. The CD30/Fc fusion protein was purified by affinity chromatography. Briefly, one liter of culture supernatant containing the CD30/Fc fusion protein was purified by filtering the supernatants (e.g., in a 0.45μ filter) and applying the filtrate to a protein G affinity column (Schleicher and Schuell, Keene, NH) according to manufacturer's instructions. The Fc portion of the fusion protein is bound by the Protein G on the column. Bound fusion protein was eluted from the column and the purity confirmed on a silver stained SDS gel.

EXAMPLE 2: Screening of Cell Lines for Binding of CD30

This example describes screening of certain cell lines for the ability to bind a CD30/Fc fusion protein. Those cell lines found to be capable of binding CD30/Fc were considered to be candidates for use as nucleic acid sources in the attempt to clone CD30-L.

Biotinylation of CD30/Fc Fusion Proteins

The purified CD30/Fc fusion protein prepared in Example 1 was labeled with biotin for use in screening cell lines. CD30/Fc or control human IL-4R/Fc were biotinylated as follows: 50 μg protein (200-500 μg/ml in 0.1M $NaHCO_3$ pH 8.3) was incubated with 2μg (1 mg/ml in DMSO) Biotin-X-N-hydroxysuccinimide (NHS, Calbiochem, La Jolla, CA) for 30 min at room temperature. At the end of the incubation period, the reaction mixture was microfuged through a 1 ml Sephadex G-25 (Pharmacia) desalting column and the eluate adjusted to 100 μg/ml in PBS plus 0.02%

NaN_3. Protein concentration of biotinylated CD30/Fc and hIL-4R/Fc was determined by micro-BCA assay (Pierce, Rockford, IL) with ultrapure bovine serum albumin as standard.

Flow cytometric staining with biotinylated Fc fusion proteins

Cell lines such as those identified below are screened for binding of biotinylated CD30/Fc by the following procedure. Staining of $1x10^6$ cells was carried out in round-bottomed 96-well microtiter plates in a volume of 20 µl. Cells were pre-incubated for 30 min at 4°C with 50 µl blocking solution consisting of 100 µg/ml human IgG1 + 2% goat serum in PBS + azide to prevent non-specific binding of labeled fusion proteins to Fc receptors. 150 µl PBS + azide was then added to the wells and cells were pelleted by centrifugation for 4 min at 1200 rpm. Pellets were resuspended in 20 µl of 5 µg/ml biotinylated CD30/Fc or biotinylated hIL-4R/Fc (as a specificity control) diluted in blocking solution. After 30-45 min incubation at 4°C, cells were washed X2 in PBS + azide and resuspended in 20 µl streptavidin-phycoerythrin (Becton Dickinson) diluted 1:5 in PBS + azide. After an additional 30 min, cells are washed x2 and are ready for analysis. If necessary, stained cells can be fixed in 1% formaldehyde, 1% fetal bovine serum in PBS + azide and stored at 4°C in the dark for analysis at a later time.

Streptavidin binds to the biotin molecule which was attached to the CD30/Fc protein. Phycoerythrin is a fluorescent phycobiliprotein which serves as a detectable label. The level of fluorescence signal was then measured for each cell type using a FACScan® flow cytometer (Becton Dickinson).

Cell Lines to be Screened for CD30/Fc Binding

Sheep red blood cell (SRBC)-specific helper T-cell lines designated 7C2 (TH1), 7B9 (TH0) and SBE11 (TH2) were derived by limiting dilution from primary antigen-induced cultures of murine C57BL/6 spleen cells. TH phenotypes of these clones were determined by their ability to secrete IL-2 and/or IL-4 in response to stimulation with the mitogen concanavalin A (ConA).

Human peripheral blood T-cells were stimulated for 16 hours with 10 µg/ml of an anti- CD3 monoclonal antibody immobilized on plastic, prior to assay for CD30/Fc binding. The anti-CD3 MAb stimulates the T-cells through the CD3-T-cell receptor (TCR) complex.

Biotinylated CD30/Fc binding

Murine T-cell lines 7C2, 7B9 and SBE11 showed significant binding of biotinylated CD30/Fc over that seen with control IL-4R/Fc, after stimulation for 18 hours with 3 μg/ml Con A. 7C2 cells were also assayed after 6 hours stimulation with Con A, and specific binding of labeled CD30/Fc was seen. The anti-CD3 MAb activated human T-cells showed significant binding of biotinylated CD30/Fc. Binding of biotinylated CD30/Fc was not detected on any of these cell lines in the absence of stimulation.

Any of the cell lines that demonstrated binding of CD30/Fc may be used as a source of nucleic acid in an attempt to isolate a CD30-L-encoding DNA sequence. A cDNA library may be prepared from any of the three Con A stimulated murine T-cell lines or the activated human peripheral blood T-cells, and screened to identity CD30-L cDNA using the direct expression cloning strategy described below, for example. Other types of activated T-cells may be screened for CD30 binding to identify additional suitable nucleic acid sources. The cells may be derived from human, murine, or other mammalian sources, including but not limited to rat, bovine, porcine, or various primate cells. Further, the T-cells may be stimulated with mitogens other than ConA or otherwise activated by conventional techniques. It is to be noted that human CD30/Fc was successfully employed to screen both human and murine cell lines in the foregoing assay (i.e., human CD30/Fc binds to a ligand on both the human and the murine cell lines tested).

EXAMPLE 3: Preparation of cDNA Library Derived from Activated Murine Helper T-cells

This example describes preparation of a cDNA library for expression cloning of murine CD30-L. The library was prepared from the murine helper T-cell line designated 7B9 (described above and in Mosley et al., *Cell* 59:335, 1989), which was stimulated for 6 hours with 3 μg/ml Con A. The library construction technique was substantially similar to that described by Ausubel et al., eds., *Current Protocols In Molecular Biology*, Vol. 1, (1987). Briefly, total RNA was extracted from the 7B9 cell line and poly $(A)^+$ mRNA was isolated by oligo dT cellulose chromatography. Double-stranded cDNA was made substantially as described by Gubler et al., *Gene* 25:263, 1983. Poly$(A)^+$ mRNA fragments were converted to RNA-cDNA hybrids by reverse transcriptase using random hexanucleotides as primers. The RNA-cDNA hybrids were then converted into double-stranded cDNA fragments using RNAase H in combination with DNA polymerase I. The resulting double-stranded cDNA was blunt-ended with T4 DNA polymerase.

Unkinased (i.e. unphosphorylated) *Bgl*II adaptors:

5'- GATCTGGCAACGAAGGTACCATGG -3'

ACCGTTGCTTCCATGGTACC -5'

were ligated to 5' ends of the resulting blunt-ended cDNA, using the adaptor cloning method described in Haymerle et al., *Nucleic Acids Res.* 14:8615, 1986. Only the 24-mer oligonucleotide (top strand) will covalently bond to the cDNA during the ligation reaction. Non-covalently bound adaptors (including the 20-mer oligonucleotide above) were removed by gel filtration chromatography at 68°C. This left 24 nucleotide non-self-complementary overhangs on cDNA. The cDNA was inserted into pDC202, a mammalian expression vector that also replicates in *E. coli*. pDC202 is derived from pDC201 (Sims et al., *Nature* 241:585, 1988). The plasmid pCD201 was assembled from (i) the SV40 origin of replication, enhancer, and early and late promoters; (ii) the adenovirus-2 major late promoter and tripartite leader; (iii) SV40 polyadenylation and transcription termination signals; (iv) adenovirus-2 virus-associated RNA genes (VAI and VAII); and (v) pMSLV (Cosman et al., *Nature 312*:768, 1984). The multiple cloning site contains recognition sites for *Kpn* I, *Sma* I, and *Bgl* II. Certain extraneous vector sequences bordering the VA genes were excised from pDC201 to create pDC202. Each of the above-named features of pDC201 is present in pDC202 as well.

pDC202 was digested with *Bgl*II and *Bgl* II adaptors were ligated thereto as described for the cDNA above, except that the bottom strand of the adaptor (the 20-mer) is covalently bound to the vector, rather than the 24-mer ligated to the cDNA. A single-stranded extension complementary to that added to the cDNA thus was added to the *Bgl*II-digested vector. The 5' ends of the adaptored vector and cDNA were phosphorylated and the two DNA species were then ligated in the presence of T4 polynucleotide kinase. Dialyzed ligation mixtures were electroporated into *E. coli* strain DH5α and transformants selected on ampicillin plates.

To create an expression cloning library, the recombinant vectors containing 7B9-derived cDNA were transferred from *E. coli* to mammalian host cells. Plasmid DNA was isolated from pools of transformed *E. coli* and transfected into a sub-confluent layer of COS-7 cells using standard techniques. The transfected cells were cultured for two to three days on chambered glass slides (Lab-Tek) to permit transient expression of the inserted DNA sequences.

EXAMPLE 4: Isolation of Murine CD30-L cDNA

This example describes screening of the expression cloning library made in Example 3 with a labeled CD30/Fc fusion protein. The purified CD30/Fc fusion protein prepared in Example 1 was radioiodinated with ^{125}I using a commercially available solid phase agent (IODO-GEN, Pierce). In this procedure, 5 μg of IODO-

GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for twenty minutes at 4° C with 75 µl of 0.1 M sodium phosphate, pH 7.4 and 20 µl (2 mCi) $Na^{125}I$. The solution was then transferred to a second glass tube containing 5 µg of CD30/Fc in 45 µl PBS and this reaction mixture was incubated for twenty minutes at 4° C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex® G-25 (Sigma), and then equilibrated in RPMI 1640 medium containing 2.5% (v/v) bovine serum albumin (BSA), 0.2% (v/v) sodium azide and 20 mM Hepes, pH 7.4 binding medium. The final pool of ^{125}I CD30/Fc was diluted to a working stock solution of 1 x 10^{-7} M in binding medium, which may be stored for up to one month at 4° C without detectable loss of receptor binding activity.

Monolayers of transfected COS-7 cells made in Example 3 were assayed by slide autoradiography for expression of CD30-L using the radioiodinated CD30/Fc fusion protein. The slide autoradiographic technique was essentially as described by Gearing et al., *EMBO J.* 8:3667, 1989. Briefly, transfected COS-7 cells were washed once with binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM Hepes pH 7.2, and 50 mg/ml nonfat dry milk) and incubated for 2 hours at 4°C in binding medium containing 1 x 10^{-9} M ^{125}I-CD30/Fc fusion protein. After incubation, cells in the chambered slides were washed three times with binding buffer, followed by two washes with PBS, (pH 7.3) to remove unbound radiolabeled fusion protein.

The cells were fixed by incubating in 10% gluteraldehyde in PBS (30 minutes at room temperature), washed twice in PBS and air-dried. The slides were dipped in Kodak GTNB-2 photographic emulsion (5x dilution in water) and exposed in the dark for two to four days days at room temperature in a light-proof box. The slides were developed in Kodak D19 developer, rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined under a microscope at 25-40x magnification. Positive slides showing cells expressing CD30-L were identified by the presence of autoradiographic silver grains against a light background.

Eight pools, each containing approximately 2000 individual clones, were identified as positive for binding the CD30/Fc fusion protein. Two pools were titred and plated to provide plates containing approximately 200 colonies each. A replica of each breakdown pool was made and the cells were scraped to provide pooled plasmid DNA for transfection into COS-7 cells. The smaller pools were screened by slide autoradiography as described previously. Several of the breakdown pools contained clones that were positive for CD30-L as indicated by the presence of an expressed gene product capable of binding to the CD30/Fc fusion protein.

Individual colonies from two of the breakdown pools were picked from the replicas and inoculated into culture medium in individual wells of 96-well plates.

Cultures were mixed by pooling rows and columns and the mixed cultures were used to prepare DNA for a final round of transfection and screening. An intersection of a positive row and and a positive column identified the positive colony. DNA from the pure clone was isolated, retransfected and rescreened.

The recombinant plasmid containing murine CD30-L cDNA was recovered from the pure clone (COS-7 host cells) and transformed into *E. coli* strain DH5α. The mammalian expression vector pDC202 containing murine CD30-L cDNA (designated pDC202-mCD30-L) was deposited in *E. coli* strain DH5α host cells with the American Type Culture Collection, Rockville, MD (ATCC) on May 28, 1992, under accession number ATCC 69004. The deposit was made under the terms of the Budapest Treaty.

A DNA sequence for the coding region of the cDNA insert of clone pDC202-mCD30-L is presented in Figure 3, along with the encoded amino acid sequence. The protein comprises an N-terminal cytoplasmic domain (amino acids 1-27), a transmembrane region (amino acids 28-48), and an extracellular, i.e., receptor-binding domain (amino acids 49-220). This protein lacks a signal peptide.

Six amino acid triplets constituting N-linked glycosylation sites are found at amino acids 56-58, 67-69, 95-97, 139-141, 175-177, and 187-189 of figure 3. The protein comprises no KEX2 protease processing sites.

In this particular vector construction, an ATG codon located in the Bgl II adaptors (see Example 3) is in the same reading frame as the CD30-L cDNA insert. Thus, a percentage of the transcripts may comprise the following DNA sequence upstream of the sequence of Figure 3. The encoded amino acids are also shown, and would be fused to the N-terminus of the Figure 3 sequence, but are not CD30-L-specific amino acids.

ATG GGC TGT GGG GCT CCT TCC CCT GAC CCA GCC
Met Gly Cys Gly Ala Pro Ser Pro Asp Pro Ala

EXAMPLE 5: Monoclonal Antibodies Directed Against CD30-L

This example illustrates the preparation of monoclonal antibodies to CD30-L. CD30-L is expressed in mammalian host cells such as COS-7 or CV1-EBNA cells and purified using CD30/Fc affinity chromatography as described herein. Purified CD30-L can be used to generate monoclonal antibodies against CD30-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. The immunogen may comprise a protein (or fragment thereof, such as the extracellular domain) fused to the peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (Hopp et al., *Bio/Technology* 6:1204, 1988 and U.S. Patent No. 5,011,912) or fused to the Fc portion of an antibody, as described above.

Briefly, mice are immunized with CD30-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional CD30-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot blot assay or ELISA (Enzyme-Linked Immunosorbent Assay), for CD30-L antibodies.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of CD30-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line (e.g., NS1 or Ag 8.653). The latter myeloma cell line is available from the American Type Culture Collection as P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified CD30-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol. 144*:4212, 1990). Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-CD30-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to CD30-L.

EXAMPLE 6: Isolation of Human CD30-L cDNA

This example illustrates a cross-species hybridization technique which was used to isolate a human CD30-L cDNA using a probe derived from the sequence of murine CD30-L. A murine CD30-L probe was produced by excising the entire cDNA insert from murine clone pDC202-mCD30-L (ATCC 69004, described in Example 4) by *Bgl* II digestion, and ^{32}P-labeling the fragment using random primers (Boehringer-Mannheim).

A human peripheral blood lymphocyte (PBL) cDNA library was constructed in a phage vector (λgt 10). The PBL cells were obtained from normal human volunteers

and treated with 10 ng/ml of OKT3 (an anti-CD3 antibody), and 10 ng/ml of human IL-2 (Immunex, Seattle, WA) for six days. The PBL cells were washed and stimulated with 500 ng/ml ionomycin (Calbiochem) and 10 ng/ml PMA (Sigma) for four hours. Messenger RNA was isolated from the stimulated PBL cells. cDNA synthesized on the mRNA template was packaged into λgt 10 phage vectors (Gigapak® Stratagene, San Diego, CA) according to manufacturer's instructions. Recombinant phage were then plated on *E. coli* strain KW251 and screened using standard plaque hybridization techniques.

The murine probe was hybridized to phage cDNA in the following hybridization buffer at 37°C overnight:

50% Formamide
20 mM Pipes (pH 6.4)
0.8 M NaCl
2 mM EDTA
0.5% SDS
0.1 mg/ml salmon sperm DNA

Hybridization was followed by washing with 2X SSC, 0.1% SDS at 50°C. Positive (hybridizing) plaques were visualized by autoradiography.

Six of the positive plaques were purified and the inserts were isolated by PCR amplification using oligonucleotides that flank the cloning site. A partial amino acid sequence for human CD30-L was derived by determining the nucleotide sequence of a portion of one of these inserts (clone #9, about 2.0 kb in length). This partial amino acid sequence is presented and aligned with the corresponding portion of murine CD30-L in Figure 4. The human sequence is in the top rows, indicated by (h), and the murine sequence is indicated by (m), with amino acids of uncertain identity being represented as X. The transmembrane region is underlined for the mouse sequence and overlined for the human sequence.

The first X (at position 6) in the human sequence is most likely a methionine residue encoded by an initiation codon. As can be seen by reference to the murine sequence of Figure 3, an N-terminal fragment (amino acids 1-130) of murine CD30-L is aligned with the partial human sequence in Figure 4.

The DNA sequence of the entire coding region of the human CD30-L clone was determined and is presented in Figure 5, along with the encoded amino acid sequence. The N-terminal cytoplasmic domain (amino acids 1 to 21) is followed by a transmembrane region (amino acids 22 to 43, underlined in Figure 5) which is followed by the extracellular, i.e., receptor-binding domain (amino acids 44-215). This protein lacks a signal peptide. Where the partial human CD30-L of Figure 4 differs from the

full length human sequence presented in Figure 5, the Figure 5 sequence is considered to be accurate. Comparison of the murine (Figure 3) and human (Figure 5) CD30-L amino acid sequences using the above-described GAP computer program reveals 73% identity and 83% similarity between the two sequences.

Amino acid triplets that constitute potential N-linked glycosylation sites are found at positions 62-64, 90-92, 134-136, 170-172, and 182-184. A KEX2 protease processing site is found at amino acids 72-73 of Figure 5. If desired, these N-glycosylation processing sites may be inactivated to preclude glycosylation as described above. The KEX2 sites may be inactivated to reduce proteolysis when the CD30-L protein is expressed in yeast cells, as described above.

The products of the above-described PCR reaction (by which the cDNA insert of the positive clone was amplified) were digested with *EcoRI* and ligated into an *EcoRI*-digested vector designated pGEMBL. Plasmid pGEMBL is a derivative of the standard cloning vector pBR322 and contains a polylinker having a unique *EcoRI* site along with several other unique restriction sites. The plasmid also comprises an ampicillin resistance gene. An exemplary vector of this type is described by Dente et al., (*Nucl. Acids Res.* 11:1645, 1983).

E. coli strain DH5α was transformed with the ligation mixture and transformants containing the desired recombinant plasmid were identified. Samples of *E. coli* DH5α containing plasmid hCD30-L/pGEMBL were deposited with the American Type Culture Collection, Rockville, MD (ATCC) on June 24, 1992, under accession number ATCC 69020. The deposit was made under the terms of the Budapest Treaty. The deposited recombinant plasmid contains human CD30-L DNA that includes the complete coding region shown in Figure 5.

EXAMPLE 7: Isolation of Murine and Human CD30-L DNA Encoding Additional N-Terminal Amino Acids

Because the CD30-L clones isolated in examples 4 and 6 had relatively short 5' noncoding regions and lacked stop codons upstream of the first initiation codon, isolation of CD30-L DNA comprising additional 5' sequences was attempted. An anchored PCR technique was employed, generally as described by Loh et al., *Science 243*:217 (1989) and Carrier et al., *Gene 116*:173 (1992), both of which are hereby incorporated by reference. The same procedures were employed for isolating murine and human clones, except as noted.

First strand cDNA was synthesized using a Superscript® cDNA kit (GIBCO/BRL, Gaithersburg, MD) on the following mRNA templates:

murine: 5μg total RNA from 7B9 cell line described in Example 3.

human: 2μg poly A^+ RNA from human peripheral blood T-cells (the stimulated PBLs described in Example 6)

The primers employed in the cDNA synthesis (referred to as primers #1 hereinafter) were:

murine: 5' AGATGCTTTGACACTTG 3'

human: 5' ATCACCAGATTCCCATC 3'

Murine primer #1 is complementary to nucleotides 265-281 of Figure 3. Human primer #1 is complementary to nucleotides 325-341 of Figure 5.

The reaction mixture was treated with RNAse H, then purified over a Sephadex G50 spin column (Sigma). After drying, the cDNA was resuspended in: 10 µl H_2O, 4 µl 5X terminal deoxynucleotidyl transferase (TdT) buffer (as specified by GIBCO/BRL, Gaithersburg, MD), 4 µl 1mM dATP, and 1 µl TdT (15 units/µl). This reaction mixture was incubated at 37°C for 10 minutes to add a poly-A tail to the 3' end of the cDNA. The reaction was stopped by heating at 68°C for 15 minutes, and the mixture was applied to a Sephadex G50 spin column. The eluate was diluted to 250 µl with 10 mM Tris (pH 7.5), 1 mM EDTA. A first PCR reaction mixture was prepared by combining:

10 µl	first strand cDNA (tailed with adenines)
10 µl	10 X buffer
2 µl	1st anchoring primer: 5' GCATGCGCGCGGCCGCGGAGGT$_{17}$ 3' (100 ng/λ)
1 µl	2nd anchoring primer: 5' GCATGCGCGCGGCCGCGGAGGTT 3' (100 ng/λ)
2 µl	primer #2 (antisense) murine: 5' ACAGAAGAGATCCTCTG 3' human: 5' CCAACACCATAATAGTG 3'
1 µl	Taq DNA polymerase
0.8 µl	25 mM dNTP's
73.2 µl	dH_2O
100.0 µl	TOTAL

The following reaction conditions (temperature cycles) were employed for this first PCR, and each of the PCRs described below:

94°C - 5 minutes - 1X

94°C - 0.5 minutes |
55°C - 1.5 minutes | - 30X
72°C - 2.5 minutes |

72°C - 5 minutes - 1X

The first anchoring primer contains a poly T segment that will anneal to the poly A tail added to the cDNA. This primer also inserts a NotI restriction site (underlined) into the amplified DNA. The second anchoring primer anneals (in later cycles of the reaction) to the NotI site-containing sequence inserted into the amplified DNA via the first anchoring primer.

The murine primer #2 is complementary to nucleotides 206-222 of figure 3. The human primer #2 is complementary to nucleotides 108-124 of figure 5.

A second PCR reaction mixture was prepared by combining:

25 µl	first PCR reaction mixture (after the above reaction)
2 µl	2nd anchoring primer
2 µl	primer #2
10 µl	10x buffer
0.8 µl	25 mM dNTPs
1 µl	Taq DNA polymerase
59.2 µl	dH_2O
100.0 µl	TOTAL

A third PCR reaction mixture was prepared by combining:

10 µl	2nd PCR reaction mixture (after completion of the reaction)
10 µl	10x buffer
2 µl	2nd anchoring primer
2 µl	primer #3
	murine: 5' GGGTCGACACTTGTGCTTCTCCAGGG 3'
	human: 5' GGGTCGACCAAGGCACAGAGCCA 3'
1 µl	Taq DNA polymerase
0.8 µl	25 mM dNTPs
74.2 µl	dH_20
100.0 µl	TOTAL

The murine primer #3 contains a segment complementary to nucleotides 49-66 of figure 3. Human primer #3 contains a segment complementary to nucleotides 80-94 of figure 5. Each primer #3 also contains a segment that introduces a SalI restriction site (underlined) into the amplified DNA.

PCR reaction products (from PCR reaction no. 2 for human and no. 3 for murine) were separated by electrophoresis on a 1% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). A PCR band comprising DNA of about 300 bp was isolated for both murine and human. The CD30-L DNA was further amplified in another PCR reaction. The reaction mixture comprised:

5 µl	band from gel (melted at 68°C)
10 µl	10x buffer
2 µl	2nd anchoring primer
2 µl	primer #3
1 µl	Taq DNA polymerase
0.8 µl	25 mM dNTP's
79.2 µl	dH_2O
100.0 µl	TOTAL

The nucleotide sequence of the reaction products was determined. The reaction products may be sequenced directly or subcloned by digesting with NotI/SalI prior to sequencing. Sequencing revealed additional DNA at the 5' end, compared to the clones of examples 4 and 6, including DNA encoding an additional 19 N-terminal amino acids for both murine and human CD30-L. DNA and encoded amino acid sequences for the

coding region of CD30-L DNA comprising this additional 5' coding sequence are shown in figures 6 (murine) and 7 (human). The additional N-terminal amino acids comprise no N-glycosylation or KEX2 protease processing sites.

The murine and human CD30-L DNAs isolated in this example were expressed in CV1-EBNA cells. The molecular weight of the expressed protein, analyzed by non-reducing SDS-PAGE, was about 26,519 daltons for murine and 26,017 daltons for human CD30-L.

Although the murine and human CD30-L proteins encoded by the clones of examples 4 and 6, respectively, are truncated at the N-terminus, the encoded proteins are biologically active in that they bind to CD30. Thus, CD30-L proteins lacking from one to all of the first 19 amino acids shown in figures 6 or 7 are biologically active CD30-L proteins of the present invention. Deletion of the first 19 amino acids of figures 6 and 7 yields an amino acid sequence identical to that presented in figures 3 and 5, respectively.

EXAMPLE 8: Analysis of Biological Activities of CD30-L

Cells on which CD30 expression has been previously observed were screened for a response to the recombinant CD30 ligand. The human cell types screened included activated T cells, three Hodgkin's lymphoma lines resembling H-RS cells with primitive B or T cell-like phenotypes, and a non-Hodgkin's lymphoma line of the large cell anaplastic lymphoma (LCAL) type.

Peripheral blood T-lymphocyte (PBT) cells were isolated by centrifugation over Histopaque (Sigma Chemical Co., St. Louis, MO) and rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes as described (Armitage et al., *Int. Immunol.* 2:1039 (1990)). The purified PBT were then cultured for 5 days in the presence of immobilized CD3 antibody and a titration of fixed CV1/EBNA cells expressing full length (membrane-bound) recombinant human CD30 ligand. In contrast to control cells transfected with vector alone, cells expressing CD30-L induced proliferation of the stimulated T cells in a dose-dependent manner, with a maximal response observed with 2.5×10^4 CV1/EBNA cells/well. This enhanced proliferation (and other activities described below) could be blocked by the inclusion of 10 µg/ml of soluble CD30/Fc. A similar ability to induce proliferation of CD3-activated T cells was seen in the presence of immobilized anti-CD30 monoclonal antibody M44, suggesting the bivalent antibody mimics ligand-induced receptor cross linking. The M44 monoclonal antibody is a mouse IgG1 generated with purified CD30-Fc as immunogen. No response was seen to CD30-L in the absence of CD3 co-stimulation.

The biological activity of CD30-L on human lymphoma cell lines known to express CD30 was investigated. The CD30$^+$ human lymphoma lines tested included HDLM-2, KM-H2, L-428, and Karpas 299 cells. Culture conditions for these four cell lines are published (Drexler et al., *Leuk. Res.* 10:487 (1986); Gruss et al., *Cancer Res.* 52:3353 (1992)).

The HD-derived cell line HDLM-2 was established from a malignant pleural effusion of a 74-year-old male with endstage IVB HD (Drexler et al., 1986, *supra*; Gruss et al., 1992, *supra*). HDLM-2 is phenotypically T-cell-like (Gruss et al., 1992, *supra*). KM-H2 and L-428 are B cell-like, HD-derived lymphoma lines. The human Karpas 299 cell line was established from blast cells in the peripheral blood of a 25-year-old white male with the diagnosis of a large cell anaplastic lymphoma (Ki-1 positive high-grade human lymphoma). The peripheral blast cells with pleomorphic nuclei resembled primitive histiocytes, which bear the surface markers CD4, CD5, HLA-DR and CD30. The Karpas 299 cell line possesses the same cytochemical, immunologic, and chromosomal profile with a 2;5 translocation as the original peripheral blood blast cells of the patient (Fischer et al., *Blood* 72:234 (1988)).

The addition of CV1/EBNA cells (10,000 cells/well) expressing recombinant human CD30-L to the HD-derived cell line HDLM-2 (50,000 cells/well) resulted in enhanced proliferation, whereas addition of control CV1/EBNA cells transfected with vector alone had minimal effect. The CD30-L-induced stimulation of HDLM-2 cell proliferation was time-dependent, with a maximal 3-4-fold enhancement observed at 72 hours. Similar results were obtained using immobilized M44 antibody, and the effect was dose-dependent. Cells cultured with an isotype-matched control monoclonal antibody showed no response. Maximal enhancement of proliferation, a five-fold increase over control cultures, was detected after stimulation with 10 μg/ml of M44 for 72 hours. Here again, the M44 CD30 monoclonal antibody has agonist characteristics and mimics properties of the ligand. In contrast to the above results, we could detect no CD30-L effects on proliferation or viability of the KM-H2 or L-428 cells, even though both lines were confirmed to be CD30$^+$ by flow cytometry with M44.

A clear and dramatically different response to CD30-L was seen with the CD30$^+$ non-Hodgkin lymphoma (LCAL) line Karpas 299. The addition of either CV1/EBNA cells expressing the CD30-L or M44 antibody to Karpas 299 cells (5×10^3 cells/well) decreased the proliferation eight-fold. This effect was further analyzed with cytotoxic assays measuring ^{51}Cr-release. Both CV1/EBNA cells expressing CD30-L and M44 antibody induced specific ^{51}Cr release from these cells in a time and dose-dependent manner. At 18 hours, the specific release in response to CD30-L or M44 was 29.4% and 30.8%, respectively. The addition of CV1/EBNA cells transfected with vector alone, or of an isotype-matched control antibody, had no effect. Thus, in

contrast to the enhanced proliferative response of the Hodgkin's lymphoma-derived HDLM-2, the response of the Karpas 299 non-Hodgkin's lymphoma line to CD30-L is cell death.

EXAMPLE 9: Northern Analysis of Murine and Human CD30-L Transcripts

Various types of cells were analyzed by Northern blotting to detect CD30-L transcripts (mRNA).

Human cells

Human PBT cells, induced with a calcium ionophore, uninduced tonsillar T cells and LPS-induced monocytes all expressed a single hybridizing transcript migrating between 18 and 28 S ribosomal RNA. IL-7-treated PBT cells, PMA treated tonsillar B cells, uninduced Jurkat or LPS activated THP-1 macrophage, and GM-CSF treated monocytes did not express CD30-L. IL-1β induced low levels of CD30-L in monocytes. In addition, placental tissue, the promyelocytic HL60 line and two Burkitt's lymphoma B cell lines (Daudi and Raji) were also negative for expression of CD30-L transcripts. Thus human CD30-L expression was detected on specifically induced T cells and monocytes/macrophages.

Murine cells

These results are mirrored in the murine system. LPS stimulated bone marrow-derived macrophage, Con A activated 7F9 T cells (similar to the 7B9 murine helper T-cell line described in examples 2 and 3) and an LPS stimulated subclone of the murine thymoma EL4 (EL4 6.1) all express a single CD30-L transcript. Unstimulated EL4 6.1 and 7F9 cells, a bone marrow-derived stromal line D11 and a thymic stromal line F4, do not express CD30-L.

EXAMPLE 10: Characterization of Recombinant CD30-L

Biochemical characteristics of the recombinant, full-length cell surface forms of murine and human CD30-L were assessed by surface radioiodinating cells transiently expressing the recombinant ligands, then immunoprecipitating the ligands with CD30/Fc (and protein G) from lysates of detergent solubilized cells. Iodoacetamide (20mM) was included in lysing and immunoprecipitation buffers to inhibit potential disulfide interchange. Washed precipitates were then displayed by SDS-PAGE with phosphorimaging. Cells transfected with vector only, or cells expressing recombinant ligand but immunoprecipitated with an isotype matched control (huIgG1), showed no bands. Under reducing conditions, the dominant product for both human and murine recombinant CD30-L is a diffuse 40 kd band. As the CD30-L protein molecular weight is 26,000 Kd, extensive use of the multiple N-linked glycosylation sites in the

extracellular domains seems clear. Disulfide-linked dimers of human CD30-L appear under non-reducing conditions, and even higher oligomers, apparently disulfide-linked, are seen with murine CD30-L. Most, but not all of these are converted to monomers upon reduction. The fact that not all oligomers were converted to monomers may reflect either differential glycosylation and/or inefficient reduction.

EXAMPLE 11: Production of a Soluble Human CD30-L Fusion Protein

A soluble fusion protein comprising an antibody Fc region polypeptide joined through a peptide linker to the N-terminus of a fragment of the human CD30-L extracellular domain was produced and tested for biological activity as follows. DNA encoding a soluble human CD30-L polypeptide comprising amino acids 47 (Asp) to 215 (Asp) of Figure 5 was isolated and amplified by PCR. The PCR was conducted by conventional procedures, using as the 5' primer an oligonucleotide comprising nucleotides 139-153 of Figure 5 and a sequence containing a recognition site for BspE1. The 3' primer spanned the termination codon of CD30-L and contained the recognition sequence for Not I.

The PCR products were digested with Bsp E1 and Not I and the desired fragment was ligated into an expression vector designated pDC408, which is a derivative of the pDC406 vector described above. pDC408 had been modified to contain (in order) 5'- murine IL-7 leader sequence - FLAG® - human IgG1 Fc domain - peptide linker.

The murine IL-7 leader sequence is described in U.S. Patent 4,965,195 and the FLAG® octapeptide is described above. The Fc polypeptide is described in example 1. A peptide linker of the sequence $Gly_4SerGly_5Ser$ was employed, and the soluble CD30-L encoding DNA was inserted immediately downstream of the peptide linker, in the same reading frame. 293 cells (ATCC CRL 1573; a transformed primary human embryonal kidney cell line) were transfected with the recombinant expression vector and cultured to permit expression and secretion of the fusion protein. The expressed protein was purified on a protein A column.

The activity of the expressed protein was measured using an inhibition assay in which the binding of ^{125}I-labelled CD30/Fc protein to CD30-L expressed on the surface of transformed CV1/EBNA cells was measured. The soluble CD30-L-containing fusion protein was shown to be capable of inhibiting this binding, thus indicating its ability to bind to CD30/Fc. The measured affinity of the soluble ligand for CD30/Fc was roughly equivalent to that of CD30/Fc for the cell-bound ligand.

CLAIMS

What is claimed is:

1. An isolated DNA sequence encoding a biologically active CD30-L polypeptide, wherein said CD30-L comprises an amino acid sequence selected from the group consisting of amino acids 1-220 of figure 3, amino acids 1-215 of figure 5, amino acids 1-239 of figure 6, and amino acids 1-234 of figure 7.

2. An isolated DNA sequence encoding a soluble CD30-L polypeptide, wherein said CD30-L comprises an amino acid sequence selected from the group consisting of amino acids 49-220 of figure 3 and amino acids z-215 of figure 5, wherein z is selected from the group consisting of 44, 45, 46, and 47.

3. An isolated DNA sequence according to claim 2, wherein said DNA sequence additionally encodes an Fc polypeptide derived from an antibody fused, directly or through a peptide linker, to the N-terminus of the CD30-L polypeptide.

4. An isolated DNA capable of hybridizing to a DNA sequence of claim 1 under moderately stringent conditions, wherein said isolated DNA encodes a biologically active CD30-L.

5. An isolated DNA sequence according to claim 4, wherein said CD30-L comprises an amino acid sequence selected from the group consisting of amino acids x to 239 of figure 6, wherein x is 1-19, and amino acids y to 234 of figure 7, wherein y is 1-19.

6. An expression vector comprising a DNA sequence according to claim 1.

7. An expression vector comprising a DNA sequence according to claim 2.

8. An expression vector comprising a DNA sequence according to claim 3.

9. An expression vector comprising a DNA sequence according to claim 4.

10. A process for preparing a CD30-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 6 under conditions promoting expression of CD30-L, and recovering the CD30-L polypeptide.

11. A process for preparing a CD30-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 7 under conditions promoting expression of CD30-L and recovering the CD30-L polypeptide.

12. A process for preparing a soluble CD30-L/Fc fusion protein, comprising culturing a host cell transformed with a vector according to claim 8 under conditions promoting expression of CD30-L/Fc, and recovering the CD30-L/Fc polypeptide.

13. A process for preparing a CD30-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 9 under conditions promoting expression of CD30-L, and recovering the CD30-L polypeptide.

14. A substantially homogeneous purified biologically active CD30-L protein, wherein said CD30-L is selected from the group consisting of murine CD30-L comprising the N-terminal amino acid sequence Met-Gln-Val-Gln-Pro-Gly-Ser-Val-Ala-Ser-Pro-Trp or Met-Glu-Pro-Gly-Leu-Gln-Gln-Ala-Gly-Ser-Cys-Gly, and human CD30-L comprising the N-terminal amino acid sequence Met-His-Val-Pro-Ala-Gly-Ser-Val-Ala-Ser-His-Leu or Met-Asp Pro-Gly-Leu-Gln-Gln-Ala-Leu-Asn-Gly-Met.

15. A purified CD30-L according to claim 14, wherein said CD30-L comprises an amino acid sequence selected from the group consisting of amino acids 1-220 of figure 3, amino acids 1-215 of figure 5, amino acids 1-239 of figure 6, and amino acids 1-234 of figure 7.

16. A substantially homogeneous soluble CD30-L polypeptide, wherein said soluble CD30-L comprises an amino acid sequence selected from the group consisting of amino acids 49-220 of figure 3 and amino acids z-215 of figure 5, wherein z is selected from the group consisting of 44, 45, 46, and 47.

17. Essentially homogeneous purified biologically active CD30-L protein, wherein said CD30-L is encoded by a DNA sequence that will hybridize to the nucleotide sequence presented in figure 3 or figure 5 under moderately stringent conditions.

18. Purified CD30-L according to claim 17, wherein said CD30-L comprises an amino acid sequence selected from the group consisting of amino acids x to 239 of figure 6, wherein x is 1-19, and amino acids y to 234 of figure 7, wherein y is 1-19.

19. A fusion protein comprising a CD30-L according to claim 17, wherein said CD30-L is a soluble CD30-L, and an Fc polypeptide derived from an antibody.

20. A dimeric protein comprising two fusion proteins according to claim 19, joined by disulfide bonds between the Fc polypeptides.

21. An antibody immunoreactive with CD30-L or an immunogenic fragment of CD30-L.

22. An antibody according to claim 21 wherein said antibody is a monoclonal antibody.

23. An antisense or sense oligonucleotide that can inhibit transcription or translation of CD30-L, comprising a sequence of at least about 14 nucleotides corresponding to a DNA sequence according to claim 1 or its DNA or RNA complement.

FIGURE 1a

```
ATG CGC GTC CTC CTC GCC GCG CTG GGA CTG CTG TTC CTG GGG GCG CTA CGA GCC TTC CCA   60
Met Arg Val Leu Leu Ala Ala Leu Gly Leu Leu Phe Leu Gly Ala Leu Arg Ala Phe Pro   20

CAG GAT CGA CCC TTC GAG GAC ACC TGT CAT GGA AAC CCC AGC CAC TAC TAT GAC AAG GCT  120
Gln Asp Arg Pro Phe Glu Asp Thr Cys His Gly Asn Pro Ser His Tyr Tyr Asp Lys Ala   40

GTC AGG AGG TGC TGT TAC CGC TGC CCC ATG GGG CTG TTC CCG ACA CAG CAG TGC CCA CAG  180
Val Arg Arg Cys Cys Tyr Arg Cys Pro Met Gly Leu Phe Pro Thr Gln Gln Cys Pro Gln   60

AGG CCT ACT GAC TGC AGG AAG CAG TGT GAG CCT GAC TAC TAC CTG GAT GAG GCC GAC CGC  240
Arg Pro Thr Asp Cys Arg Lys Gln Cys Glu Pro Asp Tyr Tyr Leu Asp Glu Ala Asp Arg   80

TGT ACA GCC TGC GTG ACT TGT TCT CGA GAT GAC CTC GTG GAG AAG ACG CCG TGT GCA TGG  300
Cys Thr Ala Cys Val Thr Cys Ser Arg Asp Asp Leu Val Glu Lys Thr Pro Cys Ala Trp  100

AAC TCC TCC CGT GTC TGC GAA TGT CGA CCC GGC ATG TTC TGT TCC ACG TCT GCC GTC AAC  360
Asn Ser Ser Arg Val Cys Glu Cys Arg Pro Gly Met Phe Cys Ser Thr Ser Ala Val Asn  120

TCC TGT GCC CGC TGC TTC TTC CAT TCT GTC TGT CCG GCA GGG ATG ATT GTC AAG TTC CCA  420
Ser Cys Ala Arg Cys Phe Phe His Ser Val Cys Pro Ala Gly Met Ile Val Lys Phe Pro  140

GGC ACG GCG CAG AAG AAC ACG GTC TGT GAG CCG GCT TCC CCA GGG GTC AGC CCT GCC TGT  480
Gly Thr Ala Gln Lys Asn Thr Val Cys Glu Ala Ser Pro Glu Asn Cys Lys Glu Pro Ser  160

GCC AGC CCA GAG AAC TGC AAG GAA CCC TCC AGT GGC ACC ATC CCC CAG GCC AAG CCC ACC  540
Pro Ala Ser Pro Gly Val Ser Pro Ala Cys Ser Gly Thr Ile Pro Gln Ala Lys Pro Thr  180
```

FIGURE 1b

```
CCG GTG TCC CCA GCA ACC TCC AGT GCC AGC ACC ATG CCT GTA AGA GGG GGC ACC CGC CTC  600
Pro Val Ser Pro Ala Thr Ser Ser Ala Ser Thr Met Pro Val Arg Gly Gly Thr Arg Leu  200

GCC CAG GAA GCT GCT TCT AAA CTG ACG AGG GCT CCC GAC TCT CCC TCC TCT GTG GGA AGG  660
Ala Gln Glu Ala Ala Ser Lys Leu Thr Arg Ala Pro Asp Ser Pro Ser Ser Val Gly Arg  220

CCT AGT TCA GAT CCA GGT CTG TCC CCA ACA CAG CCA TGC CCA GAG GGG TCT GGT GAT TGC  720
Pro Ser Ser Asp Pro Gly Leu Ser Pro Thr Gln Pro Cys Pro Glu Gly Ser Gly Asp Cys  240

AGA AAG CAG TGT GAG CCC GAC TAC TAC CTG GAC GAG GCC GGC CGC TGC ACA GCC TGC GTG  780
Arg Lys Gln Cys Glu Pro Asp Tyr Tyr Leu Asp Glu Ala Gly Arg Cys Thr Ala Cys Val  260

AGC TGT TCT CGA GAT GAC CTT GTG GAG AAG ACG CCA TGT GCA TGG AAC TCC TCC CGC ACC  840
Ser Cys Ser Arg Asp Asp Leu Val Glu Lys Thr Pro Cys Ala Trp Asn Ser Ser Arg Thr  280

TGC GAA TGT CGA CCT GGC ATG ATC TGT GCC ACA TCA GCC ACC AAC TCC TGT GCC CGC TGT  900
Cys Glu Cys Arg Pro Gly Met Ile Cys Ala Thr Ser Ala Thr Asn Ser Cys Ala Arg Cys  300

GTC CCC TAC CCA ATC TGT GCA GGA GAG ACG GTC ACC AAG CCC CAG GAT ATG GCT GAG AAG  960
Val Pro Tyr Pro Ile Cys Ala Ala Glu Thr Val Thr Lys Pro Gln Asp Met Ala Glu Lys  320

GAC ACC ACC TTT GAG GCG CCA CCC CTG GGG ACC CAG CCG GAC TGC AAC CCC ACC CCA GAG 1020
Asp Thr Thr Phe Glu Ala Pro Pro Leu Gly Thr Gln Pro Asp Cys Asn Pro Thr Pro Glu  340

AAT GGC GAG GCG CCT GCC AGC ACC AGC CCC ACT CAG AGC TTG CTG GTG GAC TCC CAG GCC 1080
Asn Gly Glu Ala Pro Ala Ser Thr Ser Pro Thr Gln Ser Leu Leu Val Asp Ser Gln Ala  360
```

FIGURE 1c

```
AGT AAG ACG CTG CCC ATC CCA ACC AGC GCT CCC GTC GCT CTC TCC TCC ACG GGG AAG CCC  1140
Ser Lys Thr Leu Pro Ile Pro Thr Ser Ala Pro Val Ala Leu Ser Ser Thr Gly Lys Pro   380

GTT CTG GAT GCA GGG CCA GTG CTC TTC TGG GTG ATC CTG GTG TTG GTT GTG GTG GTC GGC  1200
Val Leu Asp Ala Gly Pro Val Leu Phe Trp Val Ile Leu Val Leu Val Val Val Val Gly   400

TCC AGC GCC TTC CTC CTG TGC CAC CGG AGG GCC TGC AGG AAG CGA ATT CGG CAG AAG CTC  1260
Ser Ser Ala Phe Leu Leu Cys His Arg Arg Ala Cys Arg Lys Arg Ile Arg Gln Lys Leu   420

CAC CTG TGC TAC CCG GTC CAG ACC TCC CAG CCC AAG CTA GAG CTT GTG GAT TCC AGA CCC  1320
His Leu Cys Tyr Pro Val Gln Thr Ser Gln Pro Lys Leu Glu Leu Val Asp Ser Arg Pro   440

AGG AGG AGC TCA ACG CAG CTG AGG AGT GGT GCG TCG GTG ACA GAA CCC GTC GCG GAA GAG  1380
Arg Arg Ser Ser Thr Gln Leu Arg Ser Gly Ala Ser Val Thr Glu Pro Val Ala Glu Glu   460

CGA GGG TTA ATG AGC CAG CCA CTG ATG GAG ACC TGC CAC AGC GTG GGG GCA GCC TAC CTG  1440
Arg Gly Leu Met Ser Gln Pro Leu Met Glu Thr Cys His Ser Val Gly Ala Ala Tyr Leu   480

GAG AGC CTG CCG CTG CAG GAT GCC AGC CCG GCC GGG GGC CCC TCG TCC CCC AGG GAC CTT  1500
Glu Ser Leu Pro Leu Gln Asp Ala Ser Pro Ala Gly Gly Pro Ser Ser Pro Arg Asp Leu   500

CCT GAG CCC CGG GTG TCC ACG GAG CAC ACC AAT AAC AAG ATT GAG AAA ATC TAC ATC ATG  1560
Pro Glu Pro Arg Val Ser Thr Glu His Thr Asn Asn Lys Ile Glu Lys Ile Tyr Ile Met   520
```

FIGURE 1d

```
AAG GCT GAC ACC GTG ATC GTG GGG ACC GTG AAG GCT GAG CTG CCG GAG GGC CGG GGC CTG  1620
Lys Ala Asp Thr Val Ile Val Gly Thr Val Lys Ala Glu Leu Pro Gly Gly Arg Gly Leu   540

GCG GGG CCA GCA GAG CCC GAG TTG GAG GAG GAG CTG GAG GCG GAC CAT ACC CCC CAC TAC  1680
Ala Gly Pro Ala Glu Pro Glu Leu Glu Glu Leu Glu Ala Asp His Thr Pro His Tyr   560

CCC GAG CAG GAG ACA GAA CCG CCT CTG GGC AGC TGC AGC GAT GTC ATG CTC TCA GTG GAA  1740
Pro Glu Gln Glu Thr Glu Pro Pro Leu Gly Ser Cys Ser Asp Val Met Leu Ser Val Glu   580

GAG GAA GGG AAA GAA GAC CCC TTG CCC ACA GCT GCC TCT GGA AAG TGA                  1788
Glu Glu Gly Lys Glu Asp Pro Leu Pro Thr Ala Ala Ser Gly Lys END                   595
```

FIGURE 2a

```
          Bgl II
GAG CCC AGA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA   48
Glu Pro Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala   16

CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC   96
Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro   32

AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG   144
Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val    48

GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG   192
Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val    64

GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG   240
Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln    80

TAC AAC AGC ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG   288
Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln    96

GAC TGG CTG AAT GGC AAG GAC TAC AAG TGC AAG GTC TCC AAC AAA GCC   336
Asp Trp Leu Asn Gly Lys Asp Tyr Lys Cys Lys Val Ser Asn Lys Ala   112

CTC CCA GCC CCC ATG CAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC   384
Leu Pro Ala Pro Met Gln Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro   128

CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC   432
Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr   144

AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGG   480
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Arg   160
```

FIGURE 2b

```
CAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC   528
His Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr   176

AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC   576
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr   192

AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC   624
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe   208

TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG   672
Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys   224

AGC CTC TCC CTG TCT CCG GGT AAA TGA   699
Ser Leu Ser Leu Ser Pro Gly Lys End   232
```

FIGURE 3a

```
ATG CAG GTG CAG CCC GGC TCG GTA GCC AGC CCC TGG AGA AGC ACG AGG CCC TGG AGA AGC    60
Met Gln Val Gln Pro Gly Ser Val Ala Ser Pro Trp Arg Ser Thr Arg Pro Trp Arg Ser    20

ACA AGT CGC AGC TAC TTC TAC CTC AGC ACC ACC GCA CTG GTG TGC CTT GTT GTG GCA GTG   120
Thr Ser Arg Ser Tyr Phe Tyr Leu Ser Thr Thr Ala Leu Val Cys Leu Val Val Ala Val    40

GCG ATC ATT CTG GTA CTG GTA GTC CAG AAA AAG GAC TCC ACT CCA AAT ACA ACT GAG AAG   180
Ala Ile Ile Leu Val Leu Val Val Gln Lys Lys Asp Ser Thr Pro Asn Thr Thr Glu Lys    60

GCC CCC CTT AAA GGA GGA AAT TGC TCA GAG GAT CTC TTC TGT ACC CTG AAA AGT ACT CCA   240
Ala Pro Leu Lys Gly Gly Asn Cys Ser Glu Asp Leu Phe Cys Thr Leu Lys Ser Thr Pro    80

TCC AAG AAG TCA TGG GCC TAC CTC CAA GTG TCA AAG CAT CTC AAC AAT ACC AAA CTG TCA   300
Ser Lys Lys Ser Trp Ala Tyr Leu Gln Val Ser Lys His Leu Asn Asn Thr Lys Leu Ser   100

TGG AAC GAA GAT GGC ACC ATC CAC GGA CTC ATA TAC CAG GAC GGG AAC CTG ATA GTC CAA   360
Trp Asn Glu Asp Gly Thr Ile His Gly Leu Ile Tyr Gln Asp Gly Asn Leu Ile Val Gln   120

TTC CCT GGC TTG TAC TTC ATC GTT TGC CAA CTG CAG TTC CTC GTG CAG TGC TCA AAT CAT   420
Phe Pro Gly Leu Tyr Phe Ile Val Cys Gln Leu Gln Phe Leu Val Gln Cys Ser Asn His   140

TCT GTG GAC CTG ACA TTG CAG CTC CTC ATC AAT TCC AAG ATC AAA AAG CAG ACG TTG GTA   480
Ser Val Asp Leu Thr Leu Gln Leu Leu Ile Asn Ser Lys Ile Lys Lys Gln Thr Leu Val   160

ACA GTG TGT GAG TCT GGA GTT CAG AGT AAG AAC ATC TAC CAG AAT CTC TCT CAG TTT TTG   540
Thr Val Cys Glu Ser Gly Val Gln Ser Lys Asn Ile Tyr Gln Asn Leu Ser Gln Phe Leu   180
```

FIGURE 3b

```
CTG CAT TAC TTA CAG GTC AAC TCT ACC ATA TCA GTC AGG GTG GAT AAT TTC CAG TAT GTG   600
Leu His Tyr Leu Gln Val Asn Ser Thr Ile Ser Val Arg Val Asp Asn Phe Gln Tyr Val   200

GAT ACA AAC ACT TTC CCT CTT GAT AAT GTG CTA TCC GTC TTC TTA TAT AGT AGC TCA GAC   660
Asp Thr Asn Thr Phe Pro Leu Asp Asn Val Leu Ser Val Phe Leu Tyr Ser Ser Ser Asp   220

TGA   663
End
```

FIGURE 4

```
1    PGDTVXHVPAGSEAS......HLGTTSRXYFYLTTXTLALCLVFTVATIM    44  (h)
              :|.:|| ||       .: .||| ||||.| .| :|||. ||.|:
1    .....MQVQPGSVASPWRSTRPWRSTSRSYFYLSTTAL.VCLVVXVAIIL    44  (m)

45   VLVVQRTDSIPNSPDNVPLKGGNCSEDLLCILKRAPFKKSWAYLQVXKHL    94  (h)
     |||||:.||.||..:..||||||||||:|.||..| ||||||||| |||
45   VLVVQKKDSTPNTTEKAPLKGGNCSEDLFCTLKSTPSKKSWAYLQVSKHL    94  (m)

95   NKTXLSWNKDGILHGVRYQDGNLVIQFPGFV.....      125  (h)
     |.| ||||.||.:||: ||||||::||||:
95   NNTKLSWNEDGTIHGLIYQDGNLIVQFPGLYFIVCQ      130  (m)
```

FIGURE 5a

```
ATG CAT GTG CCG GCG GGC TCC GTG GCC AGC CAC CTG GGG ACC ACG AGC CGC AGC TAT TTC    60
Met His Val Pro Ala Gly Ser Val Ala Ser His Leu Gly Thr Thr Ser Arg Ser Tyr Phe    20

TAT TTG ACC ACA GCC ACT CTG GCT CTG TGC CTT GTC TTC ACG GTG GCC ACT ATT ATG GTG   120
Tyr Leu Thr Thr Ala Thr Leu Ala Leu Cys Leu Val Phe Thr Val Ala Thr Ile Met Val    40

TTG GTC GTT CAG AGG ACG GAC TCC ATT CCC AAC TCA CCT GAC AAC GTC CCC CTC AAA GGA   180
Leu Val Val Gln Arg Thr Asp Ser Ile Pro Asn Ser Pro Asp Asn Val Pro Leu Lys Gly    60

GGA AAT TGC TCA GAA GAC CTC TTA TGT ATC CTG AAA AGA GCT CCA TTC AAG AAG TCA TGG   240
Gly Asn Cys Ser Glu Asp Leu Leu Cys Ile Leu Lys Arg Ala Pro Phe Lys Lys Ser Trp    80

GCC TAC CTC CAA GTG GCA AAG CAT CTA AAC AAA ACC AAG TTG TCT TGG AAC AAA GAT GGC   300
Ala Tyr Leu Gln Val Ala Lys His Leu Asn Lys Thr Lys Leu Ser Trp Asn Lys Asp Gly   100

ATT CTC CAT GGA GTC AGA TAT CAG GAT GGG AAT CTG GTG ATC CAA TTC CCT GGT TTG TAC   360
Ile Leu His Gly Val Arg Tyr Gln Asp Gly Asn Leu Val Ile Gln Phe Pro Gly Leu Tyr   120

TTC ATC ATT TGC CAA CTG CAG TTT CTT GTA CAA TGC CCA AAT AAT TCT GTC GAT CTG AAG   420
Phe Ile Ile Cys Gln Leu Gln Phe Leu Val Gln Cys Pro Asn Asn Ser Val Asp Leu Lys   140

TTG GAG CTT CTC ATC AAC AAG CAT ATC AAA AAA CAG GCC CTG GTG ACA GTG TGT GAG TCT   480
Leu Glu Leu Leu Ile Asn Lys His Ile Lys Lys Gln Ala Leu Val Thr Val Cys Glu Ser   160
```

FIGURE 5b

```
GGA ATG CAA ACG AAA CAC GTA TAC CAG AAT CTC TCT CAA TTC TTG CTG GAT TAC CTG CAG   540
Gly Met Gln Thr Lys His Val Tyr Gln Asn Leu Ser Gln Phe Leu Leu Asp Tyr Leu Gln   180

GTC AAC ACC ACC ATA TCA GTC AAT GTG GAT ACA TTC CAG TAC ATA GAT ACA AGC ACC TTT   600
Val Asn Thr Thr Ile Ser Val Asn Val Asp Thr Phe Gln Tyr Ile Asp Thr Ser Thr Phe   200

CCT CTT GAG AAT GTG TTG TCC ATC TTC TTA TAC AGT AAT TCA GAC TGA   648
Pro Leu Glu Asn Val Leu Ser Ile Phe Leu Tyr Ser Asn Ser Asp End   215
```

FIGURE 6a

```
ATG GAG CCA GGG CTG CAA CAA GCA GGC AGC TGT GGG GCT CCT TCC CCT GAC CCA GCC ATG     60
Met Glu Pro Gly Leu Gln Gln Ala Gly Ser Cys Gly Ala Pro Ser Pro Asp Pro Ala Met    20

CAG GTG CAG CCC GGC TCG GTA GCC AGC CCC TGG AGA AGC ACG AGG CCC TGG AGA AGC ACA   120
Gln Val Gln Pro Gly Ser Val Ala Ser Pro Trp Arg Ser Thr Arg Pro Trp Arg Ser Thr    40

AGT CGC AGC TAC TTC TAC CTC AGC ACC ACC GCA CTG GTG TGC CTT GTT GTG GCA GTG GCG   180
Ser Arg Ser Tyr Phe Tyr Leu Ser Thr Thr Ala Leu Val Cys Leu Val Val Ala Val Ala    60

ATC ATT CTG GTA CTG GTA GTC CAG AAA AAG GAC TCC ACT CCA AAT ACA ACT GAG AAG GCC   240
Ile Ile Leu Val Leu Val Val Gln Lys Lys Asp Ser Thr Pro Asn Thr Thr Glu Lys Ala    80

CCC CTT AAA GGA GGA AAT TGC TCA GAG GAT CTC TTC TGT ACC CTG AAA AGT ACT CCA TCC   300
Pro Leu Lys Gly Gly Asn Cys Ser Glu Asp Leu Phe Cys Thr Leu Lys Ser Thr Pro Ser   100

AAG AAG TCA TGG GCC TAC CTC CAA GTG TCA AAG CAT CTC AAC AAT ACC AAA CTG TCA TGG   360
Lys Lys Ser Trp Ala Tyr Leu Gln Val Ser Lys His Leu Asn Asn Thr Lys Leu Ser Trp   120

AAC GAA GAT GGC ACC ATC CAC GGA CTC ATA TAC CAG GAC GGG AAC CTG ATA GTC CAA TTC   420
Asn Glu Asp Gly Thr Ile His Gly Leu Ile Tyr Gln Asp Gly Asn Leu Ile Val Gln Phe   140

CCT GGC TTG TAC TTC ATC GTT TGC CAA CTG CAG TTC CTC GTG CAG TGC TCA AAT CAT TCT   480
Pro Gly Leu Tyr Phe Ile Val Cys Gln Leu Gln Phe Leu Val Gln Cys Ser Asn His Ser   160

GTG GAC CTG ACA TTG CAG CTC CTC ATC AAT TCC AAG ATC AAA AAG CAG ACG TTG GTA ACA   540
Val Asp Leu Thr Leu Gln Leu Leu Ile Asn Ser Lys Ile Lys Lys Gln Thr Leu Val Thr   180
```

FIGURE 6b

```
GTG TGT GAG TCT GGA GTT CAG AGT AAG AAC ATC TAC CAG AAT CTC TCT CAG TTT TTG CTG  600
Val Cys Glu Ser Gly Val Gln Ser Lys Asn Ile Tyr Gln Asn Leu Ser Gln Phe Leu Leu  200

CAT TAC TTA CAG GTC AAC TCT ACC ATA TCA GTC AGG GTG GAT AAT TTC CAG TAT GTG GAT  660
His Tyr Leu Gln Val Asn Ser Thr Ile Ser Val Arg Val Asp Asn Phe Gln Tyr Val Asp  220

ACA AAC ACT TTC CCT CTT GAT AAT GTG CTA TCC GTC TTC TTA TAT AGT AGC TCA GAC TGA  720
Thr Asn Thr Phe Pro Leu Asp Asn Val Leu Ser Val Phe Leu Tyr Ser Ser Ser Asp End  239
```

FIGURE 7a

```
ATG GAC CCA GGG CTG CAG CAA GCA CTC AAC GGA ATG GCC CCT CCT GGA GAC ACA GCC ATG     60
Met Asp Pro Gly Leu Gln Gln Ala Leu Asn Gly Met Ala Pro Pro Gly Asp Thr Ala Met    20

CAT GTG CCG GCG GGC TCC GTG GCC AGC CAC CTG GGG ACC ACG AGC CGC AGC TAT TTC TAT   120
His Val Pro Ala Gly Ser Val Ala Ser His Leu Gly Thr Thr Ser Arg Ser Tyr Phe Tyr    40

TTG ACC ACA GCC ACT CTG GCT CTG TGC CTT GTC TTC ACG GTG GCC ACT ATT ATG GTG TTG   180
Leu Thr Thr Ala Thr Leu Ala Leu Cys Leu Val Phe Thr Val Ala Thr Ile Met Val Leu    60

GTC GTT CAG AGG ACG GAC TCC ATT CCC AAC TCA CCT GAC AAC GTC CCC CTC AAA GGA GGA   240
Val Val Gln Arg Thr Asp Ser Ile Pro Asn Ser Pro Asp Asn Val Pro Leu Lys Gly Gly    80

AAT TGC TCA GAA GAC CTC TTA TGT ATC CTG AAA AGA GCT CCA TTC AAG AAG TCA TGG GCC   300
Asn Cys Ser Glu Asp Leu Leu Cys Ile Leu Lys Arg Ala Pro Phe Lys Lys Ser Trp Ala   100

TAC CTC CAA GTG GCA AAG CAT CTA AAC AAA ACC AAG TTG TCT TGG AAC AAA GAT GGC ATT   360
Tyr Leu Gln Val Ala Lys His Leu Asn Lys Thr Lys Leu Ser Trp Asn Lys Asp Gly Ile   120

CTC CAT GGA GTC AGA TAT CAG GAT GGG AAT CTG GTG ATC CAA TTC CCT GGT TTG TAC TTC   420
Leu His Gly Val Arg Tyr Gln Asp Gly Asn Leu Val Ile Gln Phe Pro Gly Leu Tyr Phe   140

ATC ATT TGC CAA CTG CAG TTT CTT GTA CAA TGC CCA AAT AAT TCT GTC GAT CTG AAG TTG   480
Ile Ile Cys Gln Leu Gln Phe Leu Val Gln Cys Pro Asn Asn Ser Val Asp Leu Lys Leu   160

GAG CTT CTC ATC AAC AAG CAT ATC AAA AAA CAG GCC CTG GTG ACA GTG TGT GAG TCT GGA   540
Glu Leu Leu Ile Asn Lys His Ile Lys Lys Gln Ala Leu Val Thr Val Cys Glu Ser Gly   180

ATG CAA ACG AAA CAC GTA TAC CAG AAT CTC TCT CAA TTC TTG CTG GAT TAC CTG CAG GTC   600
Met Gln Thr Lys His Val Tyr Gln Asn Leu Ser Gln Phe Leu Leu Asp Tyr Leu Gln Val   200
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FIGURE 7b

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AAC ACC ACC ATA TCA GTC AAT GTG GAT ACA TTC CAG TAC ATA GAT ACA AGC ACC TTT CCT   660
Asn Thr Thr Ile Ser Val Asn Val Asp Thr Phe Gln Tyr Ile Asp Thr Ser Thr Phe Pro   220

CTT GAG AAT GTG TTG TCC ATC TTC TTA TAC AGT AAT TCA GAC TGA   705
Leu Glu Asn Val Leu Ser Ile Phe Leu Tyr Ser Asn Ser Asp End   234
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04926

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8, 88; 435/69.3, 69.4, 69.7, 172.1, 172.3; 530/350, 387.9; 536/23.5, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: CD30, Ki-1 antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc.Nat.Acad.Sci., Volume 84, issued December 1987, A. Aruffo and B. Seed, "Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system", pages 8573-8577, entire document.	1-23
Y	Cell, Volume 63, issued 05 OCTOBER 1990, J.G. Flanagan and P.Leder, "The *kit* ligand: A cell surface molecule altered in steel mutant fibroblasts", pages 185-194, entire document.	1-23
Y	European Journal of Immunology, Volume 19, issued January 1989, O. Josimovic-Alasevic et al., "Ki-1 (CD30) antigen is released by Ki-1 positive tumor cells *in vitro* and *in vivo*", pages 157-162, entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 July 1993	Date of mailing of the international search report 04 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer LORRAINE M. SPECTOR, PH.D. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04926

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 68, issued 07 FEBRUARY 1992, H. Durkop et al., "Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's Disease", pages 421-427, entire document.	1-23
Y	Journal of Immunology, Volume 139(6), issued 15 SEPTEMBER 1987, P. Froese et al., "Biochemical characterization and biosynthesis of the Ki-1 antigen in Hodgkin-derived and virus-transformed human B and T lymphoid cell lines", pages 2081-2087, entire document.	1-23
Y	Oncogene, Volume 1, issued 1987, O. Shohat et al., "Inhibition of cell growth mediated by plasmids encoding p53 anti-sense", pages 277-283, entire document.	23
Y	Nature, Volume 350, issued 04 APRIL 1991, M.L. Riordan and J.C. Martin, "Oligonucleotide-based therapeutics", pages 442-443, entire document.	23
Y	Nature, Volume 337, issued 09 FEBRUARY 1989, D.J. Capon et al., "Designing CD4 immunoadhesins for AIDS therapy", pages 525-531, entire document.	19-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04926

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 35/14, 37/00, 37/36, 39/00; C12P 21/06; C12N 15/00; C07K 13/00, 15/00; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.8, 88; 435/69.3, 69.4, 69.7, 172.1, 172.3; 530/350, 387.9; 536/23.5, 24.5